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A pinch of salt: Response of coastal grassland plants to simulated seawater inundation treatments

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Running Head: Plant responses to simulated seawater flooding treatments

Summary

- **Background and Aims** The combination of rising sea-levels and increased storm frequency and intensity is predicted to increase the severity of oceanic storm surge events and impact of flooding on coastal ecosystems globally. Understanding how plant communities respond to this threat necessitates experiments involving plant immersion in saline water, but logistical issues and natural variation in seawater composition, mean that pure NaCl solutions or marine aquarium salts (*MS*) are widely used. Nonetheless, their comparative impact on plant ecophysiology, and thus relevance to understanding 'real-world' flooding scenarios, is unknown.
- **Methods** In the first of two experiments, we examined how six ecophysiological responses in white clover (*Trifolium repens*) varied when plants were subjected to five different inundation treatments; i.e. deionised water, natural seawater, a *MS* solution, and two NaCl solutions. In a second experiment, we examined how immersion in deionised water, *MS* solution, and natural seawater affected six European perennial herb species, three native to Spanish sand dunes, and three from British coastal grasslands.
- **Results** The two NaCl solutions induced exceptional *Trifolium* mortality, but responses varied little between *MS* and seawater treatments. In experiment 2, although leaf tissue necrosis and proline concentrations increased, and growth decreased compared to untreated controls, only one response in one species varied between *MS* and seawater treatments. Chemical speciation modelling revealed major variation in free Na^+ and Cl^- between NaCl solutions and seawater, but minor differences between *MS* and seawater.

- **Conclusions** We show that NaCl solutions are unsuitable surrogates to investigate plant response to elevated environmental salinity. Although responses to natural seawater and *MS* were consistent within species, there was notable between species variation. Consequently, the first steps to elucidating how these species-specific responses influence coastal plant community recovery following storm surge, can likely be achieved using commercial marine aquarium salts as substitutes for natural seawater.

Key Words – Coastal plants**,** Flooding; Instant Ocean, Ionic Stress; Osmotic Stress; NaCl, Salinity, Sand dunes, Sea-level rise; Storm surge

INTRODUCTION

The past, present, and likely future impacts of anthropogenic climate change (ACC) on plant species and communities are widely reported and reasonably well understood (Parmesan & Hanley, 2015). Most studies to date however, focus on the long-term, chronic impacts of ACC (e.g. elevated CO_2, variation in precipitation regimes, and temperature increase), whereas much of the environmental threat is likely to stem from stressors and disturbances linked to an increased frequency and intensity of acute, extreme events (Rahmstorf & Coumou, 2011; Vasseur *et al*., 2014). Of these, coastal flooding represents one of the most significant challenges; a combination of increased sea-surface temperatures coupled with sea-level rise is predicted to increase the frequency and severity of oceanic storm surges globally (Vousdoukas *et al*., 2016; Vitousek *et al*., 2017). As a result, many low-lying coastal areas face an increased risk of seawater inundation (Nicholls & Cazenave, 2010) with supra-littoral habitats such as sand dunes, upper salt marshes, and grasslands likely subject to periodic seawater immersion for the first time (Hoggart *et al*., 2014). Such habitats are both economically and ecologically important since they provide a natural sea defence and important refuge for many species excluded from intensive agriculture (Fisher *et al*., 2011; Duarte *et al*., 2013; Hanley *et al*., 2014). Consequently, understanding the response of coastal vegetation to any increase in the frequency and duration of seawater inundation is critical to ensure effective coastal management (Hoggart *et al*., 2014; Hanley *et al*., 2014; Christie *et al*., 2018).

The impact of freshwater flooding on plants is well understood, but in addition to soil anoxia and reduced access to atmospheric O_2 and CO_2 (Colmer & Voesenek, 2009; Perata *et al*., 2011), seawater flooding imposes additional stresses. Most obviously, this is

elevated salinity since seawater typically contains around 35 gL^{-1} (35 ‰) salt, of which chloride and sodium contribute 19 gL^{-1} and 11 gL^{-1} respectively. Together, Na^+ and Cl^- cause both osmotic (limiting the plant's ability to absorb water) and ionic (increased toxicity) stresses, although for most species, Na^+ seems to exert more obvious (certainly better studied) toxic stress than Cl^- and (Maathuis & Amtmann, 1999; Munns & Tester, 2008). As noted by Kronzucker *et al*., (2013), this stress is widely associated with a detrimental shift in cytosolic K^+/Na^+ ratios and the disruption of cellular and whole-plant potassium homeostasis by Na^+. As a general response, plants synthesise and accumulate stress metabolites (e.g. proline) and ions (i.e. K^+) to exclude or compartmentalize Na^+ and Cl^- and re-establish homeostatic function (Flowers & Colmer, 2008; Munns & Tester, 2008). Even if successfully achieved however, this likely imposes a cost on plant growth and reproductive potential (Munns & Tester, 2008; White *et al*., 2014; Hanley *et al*., 2020) with concomitant implications for subsequent population and community-level interactions. Understanding these ecophysiological and ecological responses to seawater inundation is consequently, critical to understanding post-flooding community recovery, assembly and function (Tolliver *et al*., 1997; Tate & Battaglia, 2013; Hoggart *et al*., 2014; Lantz *et al*., 2015; Hanley *et al*., 2017).

Nonetheless, remarkably few studies have examined the response of coastal plant communities and their constituent species to acute seawater flooding, likely due in part to the difficulty in conducting realistic experiments. It is for example impossible to predict exactly where and when storm surges will occur and extremely unlikely that any two flooding events would be the same. As a result, our ability to examine the 'before and after' impacts of real-world flood events in the field is extremely limited (Middleton, 2009; Lantz *et al*., 2015). Similarly, manipulative field studies where supra-littoral coastal

vegetation is experimentally flooded with seawater are rare (Tate & Batiglia, 2013); logistical and even ethical considerations are limiting. Even when achieved, most deliberately flooded sites experience long-term inundation over natural tidal cycles (Neubauer *et al.,* 2013; Hopfensperger *et al*., 2014; Masselink *et al*., 2017), rather than acute, short-duration inundation of the kind experienced in the aftermath of storms. The lack of suitable field sites and scenarios necessitates a focus on controlled 'flooding' in laboratory and greenhouse experiments using locally collected seawater (Camprubi *et al*., 2012; Hanley *et al*., 2013, 2017, White *et al*., 2014). This raises a further issue however in that even if the ratio of the major elements remains 'nearly constant' (Levington, 2001), there is marked seasonal and regional salinity variation in seawater (Dessier & Donguy, 1994; Donguy, 1994; Donguy & Meyers, 1996).

Given the most significant impact of short-duration seawater immersion on plant metabolism and physiology seems to be associated with the effects of Na^+ and Cl^- (Flowers & Colmer, 2008; Munns & Tester, 2008), the simplest experimental approach would be to use a sodium chloride solution made up to typical seawater strength (i.e. 35‰) using deionised water. In addition to Cl^- (± 55% of total chemical content) and Na^+ (± 31%) however, seawater also contains the major ions SO_4^{2-} (7.8%), Mg^{2+} (3.7%), Ca^{2+} (1.2%) and K^+ (1.1%), and minor and trace elements (together less than 0.2%) including bromine, carbon, strontium, boron, silicon, fluorine, nitrogen, phosphorous and iron (Levington, 2001). The relative concentration of many of these other elements is much more variable than Na^+ and Cl^- (Levington, 2001; Wheeler *et al*., 2016) and their impact on plant metabolism and function less clear; some, e.g. K^+, may have direct toxicological or osmotic effects while also having the potential to mitigate or amplify the impact of other elements (Flowers & Colmer, 2008).

One possible solution is to use commercially available marine aquarium salt compounds, which closely approximate typical inorganic chemical composition of seawater and offer a relatively consistent 'seawater' surrogate (Flynn *et al*., 1995; Tolliver *et al*., 1997; Mopper *et al.,* 2016). Nonetheless, some chemical seawater constituents (e.g. nitrogen and sulphur) are mobilised rapidly by biological processes and so their concentration is spatially and temporally variable (Levington, 2001). Indeed, much of the solute content of seawater is derived from organic matter (living and dead), highlighting the important biological contribution to seawater chemistry (Levington, 2001). This biological variability may impose additional impacts on terrestrial plant response to seawater inundation beyond the chemical effects alone.

The aim of this study was to elucidate how the response of common coastal plant species to simulated flooding varied according to the 'seawater' options available. Specifically, we test the hypothesis that the most commonly applied simulated seawater treatments all elicit similar plant physiological responses. In the first experiment we subjected white clover (*Trifolium repens*) to immersion in 1: (deionised) water, 2: natural seawater, 3: commercially available marine aquarium salt, 4: sodium chloride solution balanced to average oceanic salinity (hereafter *Sal*NaCl), and 5: sodium chloride solution balanced to average ionic concentration of Instant Ocean (hereafter *Ion*NaCl). We then examined subsequent mortality, plant growth, flowering, and association with N-fixing bacteria to determine whether each treatment resulted in similar, or varying plant responses. In experiment 2, we subjected six different coastal plant species to immersion in 1: deionised water, 2: natural seawater, and 3: aquarium salt solution, quantifying immediate post-inundation proline accumulation, and subsequent longer-term leaf necrosis and growth as measures of plant response.

MATERIALS AND METHODS

Plant collection and cultivation

Native to Europe, North Africa and Asia, white clover (*Trifolium repens* L. Fabaceae) is by virtue of its value as a nitrogen-fixing pasture crop, now globally distributed. In its native range however, it is a common component of coastal plant communities such as sand dunes, upper salt marshes, and grasslands (Grime *et al*., 2007). In June 2011 we collected 12 large (± 100 mm diameter), branched plant fragments with multiple rooting points from the upper section (700m from a seawall) of a grassland pasture at South Efford Marsh near Aveton Gifford, Devon, England (50°18'14"N, 03°50'59"W). All samples were taken from distinct patches separated by at least 5 m to reduce the likelihood of collecting material from the same individual (Ab-Shukor *et al*., 1988). The plant fragments were transplanted into 110 × 110 × 120 mm plastic pots containing John Innes No. 2 potting compost and cultivated in a sheltered outdoor area. See White *et al.* (2014) for full details.

In late summer 2016, we collected seeds of *Centaurea nigra* (Asteraceae), *Lotus corniculatus* (Fabaceae), and *Plantago lanceolata* (Plantaginaceae) from coastal grasslands located across southern England (Table 1). In late spring 2017 seeds of their congeners *Centaurea polycantha, Lotus creticus*, and *Plantago coronopus* were collected from sand dunes located near Zahara de los Atunes, Andalucía, Spain. Seeds of all species were collected from mature inflorescences from a minimum of 30 maternal plants, and after drying and cleaning, stored in airtight containers at room temperature until germination.

Experiment 1

In early December 2014 stolon fragments of white clover (approximately 10mm long and with discernible roots) were cut from each of eight plants and used to cultivate 24 clones from each parent. Initially planted into 50-mm diameter pots containing John Innes No. 2 compost and retained in an unheated greenhouse with natural illumination (mean daily Max 21.8 ± 0.7 °C, Min 4.3 ± 0.3 °C), in March 2015, daughter rametes were transplanted into 75 × 75 × 80 mm plastic pots containing John Innes No. 2 compost. Plants were arranged randomly on trays with capillary matting (mean daily Max 32.4 ± 1.1 °C, Min 7.4 ± 0.3 °C), and watered twice weekly to pot capacity with tap water until the start of the experiment.

Experimental Treatments

Class A volumetric glassware and glass-distilled deionised water (ddH_2O) were used for preparation of all treatments to ensure reproducibility. Approximately 30 L of seawater was collected from Wembury, Devon, England (50°19'03"N, 04°05'03"W) in mid-March and stored in large, sealed plastic containers outdoors in the dark for 74-d until use to reduce the pool of labile dissolved organic carbon compounds present. Conductivity at the time of use was 42.4 mS cm^{-1}, and salinity 34.9 ‰. Aged seawater (hereafter *SW*) was one of our five main treatment groups, along with a no-salt immersion treatment of ddH_2O (*DW*) and one using a commercially available marine aquarium salt (*MS*) 'Instant Ocean®' (Aquarium Systems, Blacksburg, Virginia, USA). *MS* solutions using Instant Ocean have been used in studies on plant response to both saltwater flooding (Tolliver *et al*., 1997; Mopper *et al.*, 2016) and increased soil salinity (Naumann *et al*., 2007, 2008), but its

effects on plant growth and physiological responses have never been compared against natural seawater.

We dissolved 33.3 gL^{-1} of Instant Ocean into deionised water to achieve a salinity of 35.1 ‰. The balance of major cations (Na^+ K^+, Ca^{2+} Mg^{2+}, Sr^{2+}) and anions (Cl^-, SO_4^{2-}) in this *MS* approximates closely seawater salts, falling within 10 % of typical seawater concentrations by mole for most of the major anions and cations, but has 5-fold higher nitrate and 50-fold higher ammonium (Atkinson & Bingman, 1997). Many trace anions (*e.g.* Cu^{2+}, Co^{2+}) are also present at low (μM) level, although these variations relate only to total concentrations and do not take into account speciation, ion pair formation, or actual bioaccessibility (Atkinson & Bingman, 1997). Different salts however, exert variable ionic charges, such that saline solutions made up from different constituent salts can have the same salinity but different ionic strength. Consequently, we prepared two different sodium chloride solutions; one the same salinity as typical seawater (*Sal*NaCl), (Atkinson & Bingman 1998), the other the same ionic strength (*Ion*NaCl), based on Debye-Hückel theory (Debye & Hückel, 1923). We prepared 25.0 L of *Sal*NaCl solution using Trace Metals Grade (>99.99 %) sodium chloride (Sigma) in ddH_2O using Class A volumetric glassware (5-L) to a final salinity of 35 ‰. A similar volume of *Ion*NaCl was prepared with the same constituents, but assuming an average seawater ionic strength of 0.7 M (*i.e.* 38.7 g NaCl/L ddH_2O). All 'salt' solutions, plus deionised water were stored in sealed, dark plastic containers in the experimental greenhouse for two days prior to use for temperature equilibration.

In early-June 2015, six established ramets were selected from each of the eight parent 'stock' plants. Each ramet, uniform in size and appearance, was assigned at random to one of the five treatment groups, or a no-immersion control treatment. In so doing, we

ensured that each treatment group received genetically identical material. Although seawater flooding following storms can persist for up to 96-hrs, a 24 h duration is typical for low-lying UK coastline habitats following tidal-surge events (Environment Agency, 2014). By immersing to pot-level (in large plastic tubs) we simulated short-term soil waterlogging; while we recognise that seawater inundation following storm-surge would likely result in shoot submergence, we were able to separate the effect of ionic imbalance in the root-zone rather than the impact of oxygen deficiency caused by full immersion that our treatments would impose.

Immediately after immersion, pots were arranged randomly on a wire mesh-topped bench inside the greenhouse; the wire mesh allowing free drainage and prevention of cross-contamination between treatment groups. 48-hr after immersion, and thereafter every two days for a further 90 d, the pots were watered to capacity (with rain water). Mean daily greenhouse temperatures during this phase of the experiment were: 36.9 °C (± 0.8) max and 13.2 °C (± 0.2) min.

Post-immersion plant response and recovery

Following immersion, one randomly selected shoot on each plant was marked at a terminal node with loosely tied cotton thread ('Stolon Growth'). This was used to quantify subsequent stolon elongation 35-d post-immersion, when we also estimated the proportion of above-ground necrotic tissue ('Necrosis'). Mortality was checked daily from the start to the end of the experiment 90-d post-immersion, when after counting the number of fully matured inflorescences present, surviving plants were harvested (late August 2015). Plants were cleaned of any adhering compost before roots and shoots were separated and oven-dried at 50 °C for 24-hr. Total dry weight biomass (roots and shoots

combined) attained during the period after immersion was taken as a measure of plant growth. We also selected the longest root branch on each plant to quantify the number of rhizobia nodules per unit root length.

The effects of 'Immersion Treatment' on 'Necrosis' and 'Stolon Growth' at 35-d-post immersion and 'Growth', 'Flowering Effort' and 'Nodules' at harvest, were examined using One-Way ANOVA; all data were Logit ($ln(x+1)$) transformed prior to analysis to ensure heterogeneity of variance, and Tukey pairwise comparisons used to locate differences between treatment means.

Experiment 2

In mid-June 2017, seeds of all six species were set to germinate in 225mm × 165mm × 50mm (covered) propagator trays containing John Innes seed compost. One week after germination, 150 individual seedlings per species were transplanted into 50mm diameter pots containing John Innes seed compost. All initial plant cultivation was conducted in a controlled growth room set at 15°C and a 12-hour day/night illumination regime. When the plants were 6 weeks old (early August), 150 individuals from each of the UK species were transplanted into 70mm × 70mm × 80mm square pots containing John Innes seed compost and moved to an elevated, outdoor 'hard standing' area on the University of Plymouth campus. A similar procedure was used for the Spanish species, except that they were transplanted into horticultural sand (Westland Horticulture Ltd, Dungannon, UK) to better simulate sediment in their native sand dune habitat.

Experimental Treatments

In early-October 2017, 119 individual plants (checked for health and similar size) of each species were allocated at random to one of three treatment groups (*DW*, *MS* or *SW*),

subdivided into 24- or 96-hrs immersion times, such that there were 17 replicate plants per treatment/immersion time combination, or a no-immersion control treatment,. Seawater was collected from Plymouth Sound, Devon, England (50°19'03"N, 04°05'03"W) in October 2017; conductivity at the time of use was 41.6mS cm^{-1}, and salinity 34.0‰. The *MS* solution using Instant Ocean was prepared to a salinity of 34‰. Immediately after immersion, pots were arranged randomly on a wire mesh-topped bench inside a greenhouse.

Post-immersion proline accumulation

Seventy-two hours after immersion, five plants per treatment/immersion time group were selected at random for proline analysis. From these, fully expanded, healthy leaves were harvested and "flash-frozen" in liquid nitrogen before storage at -80°C. Proline analysis was adapted from Shabnam *et al*., (2016). Briefly, c. 50 mg of leaves were ground in 40% v/v EtOH at a ratio of 20µl/mg of leaf material in a cold pestle and mortar. The extract was stored at 4°C overnight to allow extraction of proline before storage at -20°C. Proline standards or extract (50µl) were heated with 100µl reaction mix (1.25% w/v ninhydrin in glacial acetic acid) at 100 °C in a covered polypropylene 96 well plate for 30 minutes before centrifugation of the plate at 1300 rpm for 2 minutes. The supernatant fluid was transferred to clean plates and absorbances determined at 520 nm using an Omega Fluostar platereader (BMG Labtech).

Post-immersion plant recovery

All remaining plants were cultivated for a further 100 d, with pots watered weekly to capacity with rainwater. Mean daily greenhouse temperatures during this phase of the

experiment were: 6.1°C (± 0.03) minimum and 18.9°C (± 0.06) maximum. At 28-d post-immersion, we estimated the proportion of above-ground necrotic tissue ('Necrosis') present on each plant. Mortality was checked daily until the end of the experiment (early January 2018) when all surviving plants were harvested and processed as describe above (Experiment 1).

The effects of 'Immersion Treatment' on 'Proline', 'Necrosis', and 'Growth' were examined using One-Way ANOVA on each species; all data were $ln(x+1)$ transformed prior to analysis and Tukey pairwise comparisons used to locate differences between treatment means. Due to the relatively large number of tests generated (i.e. six per response, three responses), we adopted $P < 0.01$ to avoid Type I error.

Solute speciation modelling

Since the true levels of free ions, and ion pairs, in the solutions used here vary from the amounts of solute added (based on formation of ion pairs and precipitating minerals), it was necessary to model the chemical interactions within the solutions. In so doing, we were able to understand how the actual ion concentrations affected plants, rather than estimating effects from, e.g. total sodium added. The speciation of ions, ion pairs and precipitates *etc.* was modelled using the *MS* composition given by Atkinson & Bingman (1997) and the *SW* composition given by Nordstrom *et al.*, (1979). The PHREEQC Interactive 3.3.12 package (Parkhurst & Appelo, 1999) was used with the Lawrence Livermore National Laboratory database (llnl.dat), which is based on the EQ3/6 model of Wolery (1979). The model was run at 20 °C on the basis of 10 kg solution under test with a headspace of 100,000 L of air comprising (% *v/v*) water vapour (1.00, since experiments were conducted *c*. 1 km from the coast), carbon dioxide (0.04), oxygen

(20.95), methane (0.00018), argon (0.93), neon (0.002), helium (0.0005), balanced with nitrogen. Liquid and gas were at atmospheric pressure and the liquid was equilibrated with the headspace mixture.

RESULTS

Experiment 1

Plant mortality was exceptionally high in the *Ion*NaCl and *Sal*NaCl treatment groups where all except one individual in *Sal*NaCl died within three weeks of immersion. By contrast, no more than one plant died in any of the other treatment groups. As a result, all further analysis focussed solely on the remaining *DW*, *MS* and *SW* treatment groups. At 35-days post immersion, *Trifolium repens* exhibited increased necrosis following *MS* or *SW* treatment (Fig 1), but *DW* had no effect ($F_{3,27}$ = 12.08, P<0.001) compared to the 'no immersion' control. Stolon elongation however, did not vary between treatment groups ($F_{3,27}$ = 2.52, P= 0.079). By the end of the experiment, plants in both the *MS* and *SW* treatments were considerably smaller than those in untreated controls ($F_{3,26}$ = 5.78, P=0.004). Both 'Flowering Effort' ($F_{3,26}$ = 2.43, P=0.087) and root colonisation by rhizobia ($F_{3,26}$ = 2.14, P=0.12) were unaffected by immersion treatment (Fig 1). *Post-hoc* examination of treatment means showed no variation in plant necrosis or final biomass between *MS* and *SW* treatments (Fig 1).

Experiment 2

No more than two plants of twelve in any of the species/treatment group combinations died over the course of the experiment and we attempt no further analysis on mortality.

The effects of immersion treatments on initial proline accumulation varied between species and treatments (Fig 2). For the two *Centaurea* species, although *DW96* had no effect on leaf proline concentrations compared to untreated controls, the *MS* and *SW* immersion treatments resulted in significant accumulation (*C. nigra* – $F_{5,24}$ = 22.6, P<0.001; *C. polycantha* – $F_{6,28}$ = 4.45, P=0.003). The effect was however, more marked for *C. nigra*, where 96-hrs *MS* and *SW* immersion yielded a 3- and 5-fold respectively increase in proline concentrations (note the *DW*24 sample for this species was lost prior to analysis). For *C. polycantha*, *post-hoc* analysis suggested that *SW*24 was the only treatment to stimulate significantly increased proline synthesis, even though concentrations more than doubled in all *MS* and *SW* treatments compared to the control. *Lotus creticus* ($F_{6,28}$ = 5.43, P<0.001) exhibited a similar response to *C. nigra*, i.e. higher proline levels in the longer *MS* and *SW* immersion treatments. *Lotus corniculatus* however ($F_{6,28}$ = 5.78, P<0.001), had significant increased proline concentrations only in *MS96* and *SW96*. Neither of *Plantago lanceolata* ($F_{5,28}$ = 1.65, P=0.163) or *P. coronopus* ($F_{6,28}$ = 1.67, P=0.165) showed any variation in post-immersion proline levels. Consistent for all species, we found no variation in proline accumulation response between 'time-equivalent' *MS* or *SW* treatments.

At 28-days post immersion, all six species exhibited increased necrosis following *MS* or *SW* treatment (Fig 3); *DW* had no effect. For *Centaurea nigra* ($F_{6,77}$ = 13.01, P<0.001), immersion in *MS96* and both *SW* treatments increased leaf necrosis compared to the control, while for *C. polycantha* ($F_{6,77}$ = 17.47, P<0.001), all *MS* and *SW* treatments elicited this effect. *Lotus corniculatus* ($F_{6,77}$ = 20.68, P<0.001), was the only species exhibiting significant variation between time-equivalent (i.e. 24-hr) *MS* and *SW* treatments, where *SW24* did not vary from untreated controls. Although unaffected at

shorter durations, *L. creticus* ($F_{6,77}$ = 4.59, P=0.001) displayed more necrosis in both *IO96* and *SW96* treatments. Both *Plantago* species suffered increased necrosis following *MS* and *SW* immersion; all treatments, except *MS24*, caused increased necrosis in *P. lanceolata* ($F_{6,77}$ = 7.97, P<0.001), while for *P. coronopus* ($F_{6,77}$ = 5.27, P<0.001), elevated tissue necrosis was common throughout.

Five of the six species exhibited reduced growth (final plant dry biomass) following *MS* or *SW* treatment (Fig 4); *DW* had no effect. For both *Centaurea nigra* ($F_{6,76}$ = 20.03, P<0.001) and *C. polycantha* ($F_{6,74}$ = 16.74, P<0.001), all *MS* and *SW* treatments resulted in reduced size. *Plantago coronopus* ($F_{6,75}$ = 6.10, P<0.001), exhibited a similar response, while *P. lanceolata* ($F_{6,77}$ = 5.02, P<0.001) plants in all *MS* and *SW* treatments, except *MS96*, were smaller than controls. For the two *Lotus* species (*L. corniculatus* - $F_{6,77}$ = 6.95, P<0.001; *L. creticus* - $F_{6,73}$ = 2.77, P=0.018) however, we observed few treatment-specific effects; *L. creticus* did not achieve our P<0.01 criterion, while for *L. corniculatus*, *post-hoc* tests suggested that only plants in the *MS24* treatment were smaller than controls. Nonetheless, consistent for all six species, there was no variation in final dry biomass between 'time-equivalent' *MS* or *SW* treatments.

Solute speciation modelling

Modelling of *MS* composition compared with *SW* showed that overall available Na^+ and Cl^- concentrations were broadly similar; i.e. Instant Ocean 430 mM and 488 mM, respectively, *SW* 434 mM and 523 mM, respectively. For an NaCl solution that was salinity-matched to *MS* (i.e. *Sal*NaCl), concentrations of free Na^+ and Cl^- were substantially higher (both 572 mM), with most of the c. 25 mMol per litre that was not

present as free ions (since 596 mM total Na^+ and Cl^- added) found as the NaCl ion pair in solution. In both *SW* and *MS*, free K^+ was present at 6.3 mM and 9.0 mM, respectively; a slight increase in the key ion used by plants to re-establish homeostatic function after exposure to NaCl (Munns & Tester 2008).

DISCUSSION

Our study presents three major conclusions. First, exceptionally high *Trifolium* mortality in the *Ion*NaCl and *Sal*NaCl treatments (experiment 1) shows that 'pure' NaCl solutions are unsuitable surrogates to study the effect of seawater immersion on plant physiology. Second, except one instance (necrosis in 24-hr treatments for *Lotus corniculatus*), all *MS vs SW* comparisons suggest that a commercially available marine aquarium salt elicits similar plant ecophysiological responses to natural seawater. Finally, all species responded negatively to simulated seawater flooding (*MS* or *SW* treatments).

Although the greatest impact of seawater flooding on plant performance may stem from the ionic and osmotic stress imposed by Na^+ and Cl^-, our results suggest that other seawater constituents moderate these effects. From the methodological perspective, this is important because a number of studies have attempted to mimic the impact of salt-spray and/or sea water immersion using NaCl solutions applied directly onto the plant or soil surface (Ab-Shakor *et al*., 1988; Sykes & Wilson, 1989; van Puijenbroek *et al*., 2017; Varone *et al*., 2017). In so doing, these experiments fail to account for the likelihood that the ionic and osmotic stresses they ascribed to elevated Na^+ and Cl^- are in fact, influenced or moderated by other salts. One area for further investigation (specifically in comparison with NaCl solutions) is to determine whether K^+ in seawater (1.1% of total salt

concentration) helps mitigate deleterious changes in cytosolic K^+/Na^+ ratios and disruption of potassium homeostasis (Maathuis & Amtmann, 1999; Kronzucker *et al*., 2013). Similarly, changes in the cytoplasmic balance of Na^+/SO_4^{2-}, Na^+/Mg^{2+}, and Na^+/Ca^{2+} ratios also have deleterious effects on plants grown in high salinity, effects likely magnified when 'pure' NaCl solutions are used rather than seawater that naturally contains these SO_4^{2-}, Mg^{2+}, and Ca^{2+} ions (Maas & Grattan, 1999; Maathuis & Amtmann, 1999; Shabala *et al*., 2005). Our *Trifolium* response data (experiment 1) certainly call into question the biological relevance of the many studies that seek to assess crop plant response to increased soil salinity using NaCl solutions (e.g. Dai *et al*., 2018; Flam-Shepherd *et al.*, 2018; Wu *et al*., 2018; Zhang *et al*., 2018). Salinized irrigation waters for example, contain a range of cations and anions beyond Na^+ and Cl^- (Maas & Grattan, 1999) and our speciation modelling shows that a NaCl solution matched to average seawater salinity contains considerably more free Na and Cl ions than seawater (i.e. an increase of 32% and 9% in *Sal*NaCl respectively).

Although commercial aquarium salts have been used to determine how salinity affects coastal plants (Tolliver *et al*., 1997; Mopper *et al*., 2004; Naumann *et al*., 2008), these studies have assumed, rather than demonstrated, that observed effects were compatible with those produced by natural seawater. Our results suggest that this assumption may be valid. In comparisons of six different biochemical, growth and reproductive responses involving seven different plant species, we found only one significant difference between time-equivalent *SW* and *MS* immersion treatments; i.e. above-ground tissues necrosis in *Lotus corniculatus* was twice the amount in 24-hr *MS* immersion compared to 24-hr *SW* plants. This necrosis response seems to have carried over into final plant biomass where 24-hr *MS* was the only treatment to display significantly reduced growth in comparison

406 to the untreated control. The fact that these necrosis and biomass differences was not
407 apparent in the 96-hr treatments also suggests however, that any response is at best short-
408 lived and may even be a statistical artefact. The general consistency of observed
409 biological responses, corroborates our modelling of the compositions of *MS* and *SW* in
410 that concentrations of free Na^+ (less than 1% difference) and Cl^- (7% higher in *SW*) ions
411 are remarkably similar. In-fact given its role in counteracting cytoplasmic Na^+
412 accumulation, the (42%) higher K^+ availability in *MS* might suggest that plants subjected
413 to *MS* rather than *SW* would recover better from simulated flooding. No plant response
414 observed in our experiments corroborated this suggestion however.

415 Although in experiment 2, all six species were affected negatively by (simulated)
416 seawater immersion for at least two of the responses examined, there were some
417 interesting patterns of response. First, and as might be expected, congenerics tended to
418 react in broadly similar ways. For example, while neither *Plantago* species showed any
419 variation in leaf proline concentrations, proline responses to all immersion treatments in
420 the two *Lotus* species were remarkably similar. In *Centaurea*, necrosis and final plant
421 biomass also showed very similar treatment-specific responses. More interesting than any
422 indication of phylogenetic conservation, was perhaps the general commonality of
423 response of congenerics grown in different media (i.e. English species in commercial
424 potting compost; Spanish species in horticultural sand). When coupled with the dramatic
425 response of *Trifolium repens* to *Sal*NaCl and *Ion*NaCl solutions in experiment 1, this
426 observation suggests that achieving a field-relevant salinity treatment, is a more important
427 methodological consideration than what growing media is used to cultivate plants.
428 Second, in terms of the overall lack of plant mortality, all species showed a remarkable
429 tolerance to up to 4 days simulated seawater flooding. Finally, the consistency of all other

plant responses to *MS* and *SW* treatments nonetheless highlights the negative impact seawater flooding exerts on coastal vegetation, underscoring growing concerns about the predicted increase in the frequency and severity of oceanic storm surges on low-lying coastal areas (Nicholls & Cazenave, 2010).

An important consideration here is that all experiments were performed on plants grown in monoculture in greenhouse conditions, free from competition and environmental stressors. Indeed, even in controlled greenhouse experiments, the responses of plants to simulated seawater flooding in monoculture changed when the same species were grown together (Hanley *et al*., 2017). Consequently, even apparently minor species-specific differences in plant response to seawater inundation are likely to be magnified in sand dunes, salt marshes, and other coastal habitats following actual flood events such that species composition is modified after the event (see Engels & Jensen, 2010; Guo & Pennings, 2012; Schile *et al*., 2017). For example, a study on long-term tundra recovery following a major storm surge in the Canadian Arctic (Lantz *et al*., 2015) reported species-specific variation in plant recovery; specifically, graminoids exhibiting greater resilience than shrubs. This is important because any reduction in species diversity or loss of key plant functional groups stemming from increased flood severity or frequency may reduce community resilience to further perturbation. Ford *et al*., (2016) for example, recently described how reductions in salt marsh diversity led to increased erosion potential, particularly where sandy, low organic content soils predisposed these habitats to sediment loss. The global importance of plant communities to coastal defence, at a time when they also face increased flood risk (Duarte *et al*., 2013; Morris *et al*., 2018), gives urgency to our need to better understand how acute seawater inundation affects component species and ecosystem processes. Our inability to predict where and when

flooding will happen, and difficulties associated with conducting manipulative experiments on natural communities, means plant biologists may be constrained to work in more highly controlled systems to achieve this aim. We demonstrate here that although a pure NaCl solution is an inappropriate surrogate, commercial marine aquarium salts may offer a suitable alternative to the logistical problems and biochemical variations associated with using natural seawater.

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LITERATURE CITED

Ab-Shukor NA, Kay QON, Stevens DP, Skibinski DOF. 1988. Salt tolerance in natural populations of *Trifolium repens* L. *New Phytologist* **109:** 483-490.

Atkinson MJ, Bingman C. 1997. Elemental composition of commercial seasalts. *Journal of Aquariculture, and Aquatic* Sciences **8**: 39–43

Camprubi A, Abril M, Estaun V, Calvet C. 2012. Contribution of arbuscular mycorrhizal symbiosis to the survival of psammophilic plants after sea water flooding. *Plant and Soil* **351:** 97–105.

Christie EK, Spencer T, Owen D, McIvor AL, Möller I, Viavattene C. 2018. Regional coastal flood risk assessment for a tidally dominant, natural coastal setting: North Norfolk, southern North Sea. *Coastal Engineering* **134:** 177-190.

Colmer TD, Voesenek LACJ. 2009. Flooding tolerance: suites of plant traits in variable environments. *Functional Plant Biology* **36:** 665-681.

Dai W, Wang M, Gong X, Liu JH. 2018 The transcription factor FcWRKY40 of *Fortunella crassifolia* functions positively in salt tolerance through modulation of ion homeostasis and proline biosynthesis by directly regulating SOS2 and P5CS1 homologs. *New Phytologist* **219**: 972-989.

Debye P, Hückel E. 1923. Zur Theorie der Elektrolyte. I. Gefrierpunktserniedrigung und verwandte Escheinungen. *Physikalische Zeitschrift* **24:** 185-206.

Dessier A, Donguy JR. 1994. The sea surface salinity in the tropical Atlantic between 10°S and 30°N—seasonal and interannual variations (1977–1989). *Deep Sea Research Part I* **41:** 81–100.

Donguy J R, Meyers G. 1996. Seasonal variations of seasurface salinity and temperature in the tropical Indian Ocean. *Deep Sea Research Part I* **43:** 117–138.

Donguy JR. 1994. Surface and subsurface salinity in the tropical Pacific Ocean: Relations with climate. *Progress in Oceanography* **34:** 45–78.

Duarte CM, Losada I.J, Hendriks IE, Mazarrasa I, Marba N. 2013. The role of coastal plant communities for climate change mitigation and adaptation. *Nature Climate Change* **3:** 961-968.

Engels JG, Jensen K 2010. Role of biotic interactions and physical factors in determining the distribution of marsh species along an estuarine salinity gradient. *Oikos* **119:** 679-685.

Environment Agency UK 2014. DataShare. Available from http://www.geostore.com/environment-agency/. Accessed 4th April 2014.

Flam-Shepherd R, Huynh WQ, Coskun D, Hamam AM, Britto DT, Kronzucker HJ. 2018. Membrane fluxes, bypass flows, and sodium stress in rice: the influence of silicon. *Journal of Experimental Botany* **69**: 1679–1692.

Fisher B, Bradbury RB, Andrews JE, *et al*. 2011. Impacts of species-led conservation on ecosystem services of wetlands: understanding co-benefits and tradeoffs. *Biodiversity Conservation* **20:** 2461-2481.

Flowers TJ, Colmer TD. 2008. Salinity tolerance in halophytes. *New Phytologist* **179:** 945-963.

Flynn KM, McKee KL, Mendelssohn IA. 1995. Recovery of freshwater marsh vegetation after a saltwater intrusion event. *Oecologia* **103:** 63-72.

Ford H, Garbutt A, Ladd C, Malarkey J, Skov MW. 2016. Soil stabilization linked to plant diversity and environmental context in coastal wetlands. *Journal of Vegetation Science* **27:** 259–268.

Grime JP, Hodgson JG, Hunt R. 2007. *Comparative Plant Ecology* 2nd Edn. Dalbeattie, Scotland, Castlepoint Press.

Guo HY, Pennings SC. 2012. Mechanisms mediating plant distributions across estuarine landscapes in a low-latitude tidal estuary. *Ecology* **93:** 90-100.

Hanley ME, Yip PYS, Hoggart S, Bilton DT, Rundle SD, Thompson RC. 2013. Riding the storm: The response of *Plantago lanceolata* to simulated tidal flooding. *Journal of Coastal Conservation* **17:** 799-803.

Hanley ME, Hoggart SPG, Simmonds DJ, *et al.* 2014. Shifting sands? Coastal protection by sand banks, beaches and dunes. *Coastal Engineering* **87:** 136-146.

Hanley ME, Gove TL, Cawthray GR, Colmer TD. 2017. Differential responses of three coastal grassland species to seawater flooding. *Journal of Plant Ecology* **10:** 322–330.

Hanley ME, Hartley FC, Hayes L, Franco M. 2020. Simulated seawater flooding reduces oilseed rape growth, yield, and progeny performance. *Annals of Botany* In Press.

Hoggart SPG, Hanley ME, Parker DJ, *et al.* 2014. The consequences of doing nothing: The effects of seawater flooding on coastal zones. *Coastal Engineering* **87:** 169-182.

Hopfensperger, KN Burgin AJ, Schoepfer VA, Helton AM. 2014 Impacts of saltwater incursion on plant communities, anaerobic microbial metabolism and resulting relationships in a restored freshwater wetland. *Ecosystems* **17**: 792–807.

Kronzucker HJ, Coskun D, Schulze LM, Wong JR, Britto DT 2013. Sodium as nutrient and toxicant. *Plant Soil* **369**: 1–23.

Lantz TC, Kokelj SV, Fraser RH. 2015. Ecological recovery in an Arctic delta following widespread saline incursion. *Ecological Applications* **25:** 172–185.

Levinton JS. 2001. *Marine Biology*, 2nd Edition. OUP, Oxford.

Maathuis FJM, Amtmann AA. 1999. K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. *Annals of Botany* **84**: 123–133.

Mass EV, Grattan SR. 1999. Crop yield as affected by salinity. In: Skaggs RW, van Schilfgaarde J. (Eds.). *Agricultural drainage, agronomy monograph*. ASA, CSSA, and SSSA, Madison, WI, pp. 55–108.

Masselink G, Hanley ME, Halwyn AC, *et al.* 2017. Evaluation of salt marsh restoration by means of self-regulating tidal gate – Avon estuary, south Devon, UK. *Ecological Engineering* **106:** 174-190.

Middleton EA. 2009. Regeneration of coastal marsh vegetation impacted by hurricanes Katrina and Rita. *Wetlands* **29**, 54-65.

Mopper S, Wang Y, Criner C, Hasenstein K. 2004. *Iris hexagona* hormonal responses to salinity stress, leafminer herbivory, and phenology. *Ecology* **85:** 38–47.

Mopper S, Wiens KC, Goranova GA. 2016. Competition, salinity, and clonal growth in native and introduced irises. *American Journal of Botany* **103:** 1575–1581.

Morris RL, Konlechner TM, Ghisalberti M, Swearer SE. 2018. From grey to green: Efficacy of eco-engineering solutions for nature-based coastal defence. *Global Change Biology* doi.org/10.1111/gcb.14063.

Munns R, Tester M. 2008. Mechanisms of salt tolerance. *Annual Review of Plant Biology* **59:** 651-681.

Naumann JC, Young DR, Anderson JE. 2007. Linking leaf optical properties to physiological responses for stress detection in coastal plant species. *Physiologia plantarum* **131**: 422-433.

Naumann JC, Young DR, Anderson JE. 2008. Leaf chlorophyll fluorescence, reflectance, and physiological response to freshwater and saltwater flooding in the evergreen shrub, *Myrica cerifera*. *Environmental and Experimental Botany* **63:** 402–409.

Neubauer SC, Franklin RB, Berrier DJ. 2013. Saltwater intrusion into tidal freshwater marshes alters the biogeochemical processing of organic carbon. *Biogeosciences* **10:** 8171-8183.

Nicholls RJ, Cazenave A. 2010. Sea-level rise and its impact on coastal zones. *Science* **328:** 1517–1520.

Nordstrom DK, Plummer LN, Wigley TML, *et al.* 1979. A comparison of computerized chemical models for equilibrium calculations in aqueous solutions. In: Jenne EA (Ed) Chemical modelling in aqueous systems: Speciation, sorption, solubility, and kinetics. *American Chemical Society Symposium Series* **93:** 857-892.

Parkhurst DL, Appelo CAJ. 1999. User's guide to PHREEQC (Version 2): A computer program for speciation, batch-reaction, one-dimensional transport, and inverse geochemical calculations. *Water-Resources Investigations Report* 99-4259, U.S. Geological Survey.

Parmesan C, Hanley ME. 2015. Plants and climate change: complexities and surprises. *Annals of Botany* **115:** 849-864.

Perata P, Armstrong W, Laurentius AC, Voesenek J. 2011. Plants and flooding stress. *New Phytologist* **190:** 269–273.

Rahmstorf S, Coumou D. 2011. Increase of extreme events in a warming world. *Proceedings of the National Academy of Sciences USA* **108**: 17905–17909.

Schile LM, Callaway JC, Suding KN, Kelly NM. 2017. Can community structure track sea-level rise? Stress and competitive controls in tidal wetlands. *Ecology and Evolution* **7:** 1276–1285.

Shabala S, Shabala L, Van Volkenburgh E, Newman I. 2005. Effect of divalent cations on ion fluxes and leaf photochemistry in salinized barley leaves. *Journal of Experimental Botany* **56:** 1369–1378.

Shabnam N, Tripathi I, Sharmila P, Pardha-Saradhi P. 2016. A rapid, ideal, and eco-friendlier protocol for quantifying proline, *Protoplasm* **253**: 1577 – 1582.

Sykes MT, Wilson JB. 1989. The effect of salinity on the growth of some New Zealand sand dune species. *Acta Botanica Neerlandica* **38:** 173–182.

Tate AS, Battaglia LL. 2013. Community disassembly and reassembly following experimental storm surge and wrack application. *Journal of Vegetation Science* **24:** 46-57.

Tolliver KS, Martin DW, Young DR. 1997. Freshwater and saltwater flooding response for woody species common to barrier island swales. *Wetlands* **17:** 10-18.

van Puijenbroek MEB, Teichmann C, Meijdam N, Oliveras I, Berendse F, Limpens J. 2017. Does salt stress constrain spatial distribution of dune building grasses *Ammophila arenaria* and *Elytrichia juncea* on the beach? *Ecology and Evolution* **7**, 7290-7303.

Vasseur DA, DeLong JP, Gilbert B, *et al*. 2014. Increased temperature variation poses a greater risk to species than climate warming. *Proceedings of the Royal Society B-Biological Sciences* **281**: 1–8.

Varone L, Catoni R, Bonito A, Gini E, Gratani L. 2017. Photochemical performance of *Carpobrotus edulis* in response to various substrate salt concentrations. South *African Journal of Botany* **111**: 258-266.

Vitousek S, Barnard PL, Fletcher CH, Frazer N, Erikson L, Storlazzi CD. 2017. Doubling of coastal flooding frequency within decades due to sea-level rise. *Scientific Reports* **7**: 1399.

Vousdoukas MI, Voukouvalas E, Annunziato A, Giardino A, Feyen L. 2016. Projections of extreme storm surge levels along Europe. *Climate Dynamics* **47:** 3171–3190.

Wheeler SG, Russell AD, Fehrenbacher JS, Morgan SG. 2016. Evaluating chemical signatures in a coastal upwelling region to reconstruct water mass associations of settlement-stage rockfishes. *Marine Ecological Progress Series* **550**: 191-206

White AC, Colmer TD, Cawthray GR, Hanley ME. **2014.** Variable response of three *Trifolium repens* ecotypes to soil flooding by seawater. *Annals of Botany* **114:** 347-356.

Wolery TJ. 1979. Calculation of chemical equilibrium between aqueous solutions and minerals - The EQ3/6 software package. *Lawrence Livermore National Laboratory Report UCRL-52658*. Livermore, California, USA.

Wu H, Shabala L, Azzarello E, *et al.*, 2018. Na^+ extrusion from the cytosol and tissue-specific Na^+ sequestration in roots confer differential salt stress tolerance between durum and bread wheat. *Journal of Experimental Botany* **69**: 3987–4001

Zhang M, Cao Y, Wang Z, *et al.*, 2018. A retrotransposon in an HKT1 family sodium transporter causes variation of leaf Na^+ exclusion and salt tolerance in maize. *New Phytologist* **217**: 1161-1176.

631 **Table 1.** Details of seed collection sites for six coastal dune and grassland species from
632 SW Spain and southern England used to compare plant performance following
633 simulated seawater flooding treatments.

634

Region	Species	Site name	Lat:Long
Southern England	*Centaurea nigra* L.	Saltash, Cornwall	50°23`37``N 04°13`40``W
	Lotus corniculatus L.	Wembury, Devon	50°18`59``N 04°06`14``W
	Plantago lanceolata L.	Sandwich, Kent	51°16`48``N 01°21`42``E
South West Spain	*Centaurea polyacantha* Willd.	Atlanterra, Cadiz	36°05`39``N 05°48`44``W
	Lotus creticus L.	Zahara, Cadiz	36°08`15``N 05°51`01``W
	Plantago coronopus L.	Zahara, Cadiz	36°07`35``N 05°50`23``W

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Figures

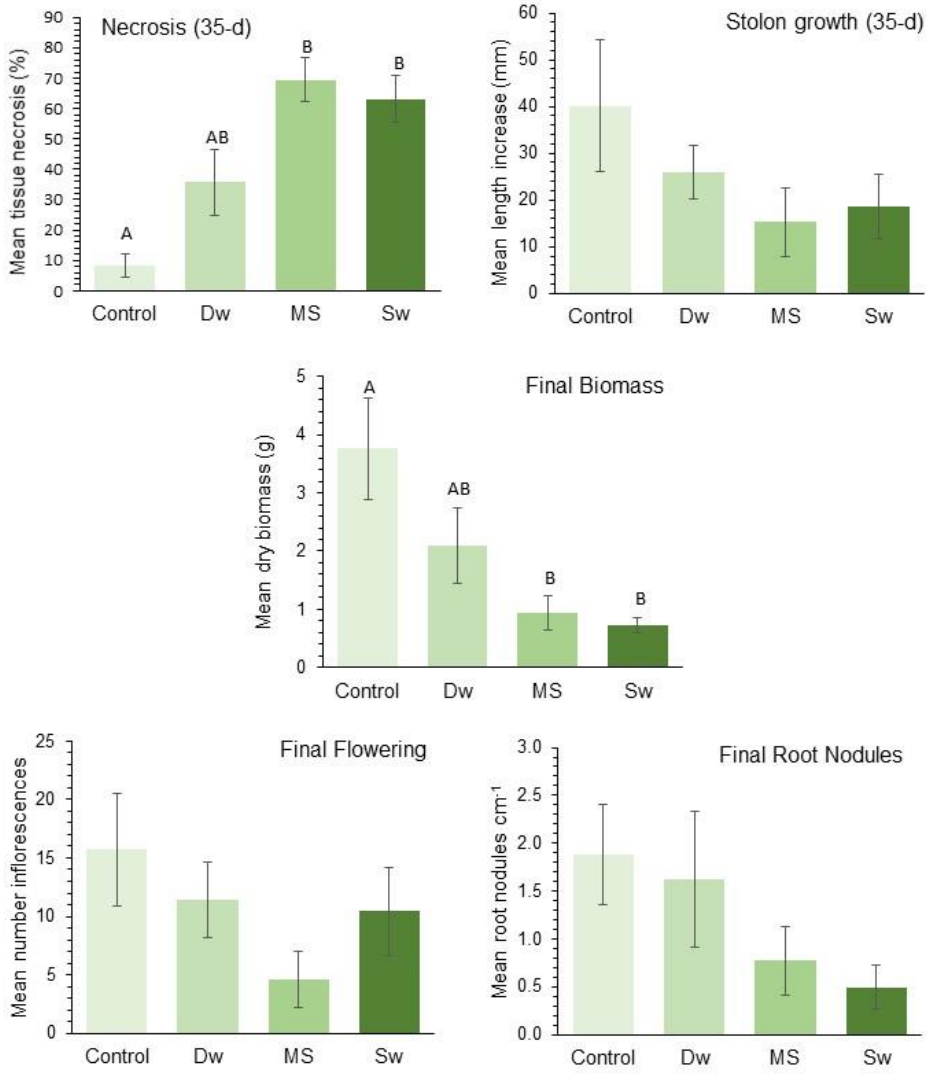

Figure 1. Responses of *Trifolium repens* to simulated seawater flooding (MS – a marine aquarium salt solution ('Instant Ocean®'); SW – natural seawater) compared with immersion in deionised water (DW) or untreated controls. Panels show effects on; above-ground tissue necrosis and stolon extension at 28-d post immersion, and final plant dry weight biomass, inflorescence number, and root colonisation by *Rhizobia* at 90-d-post immersion.

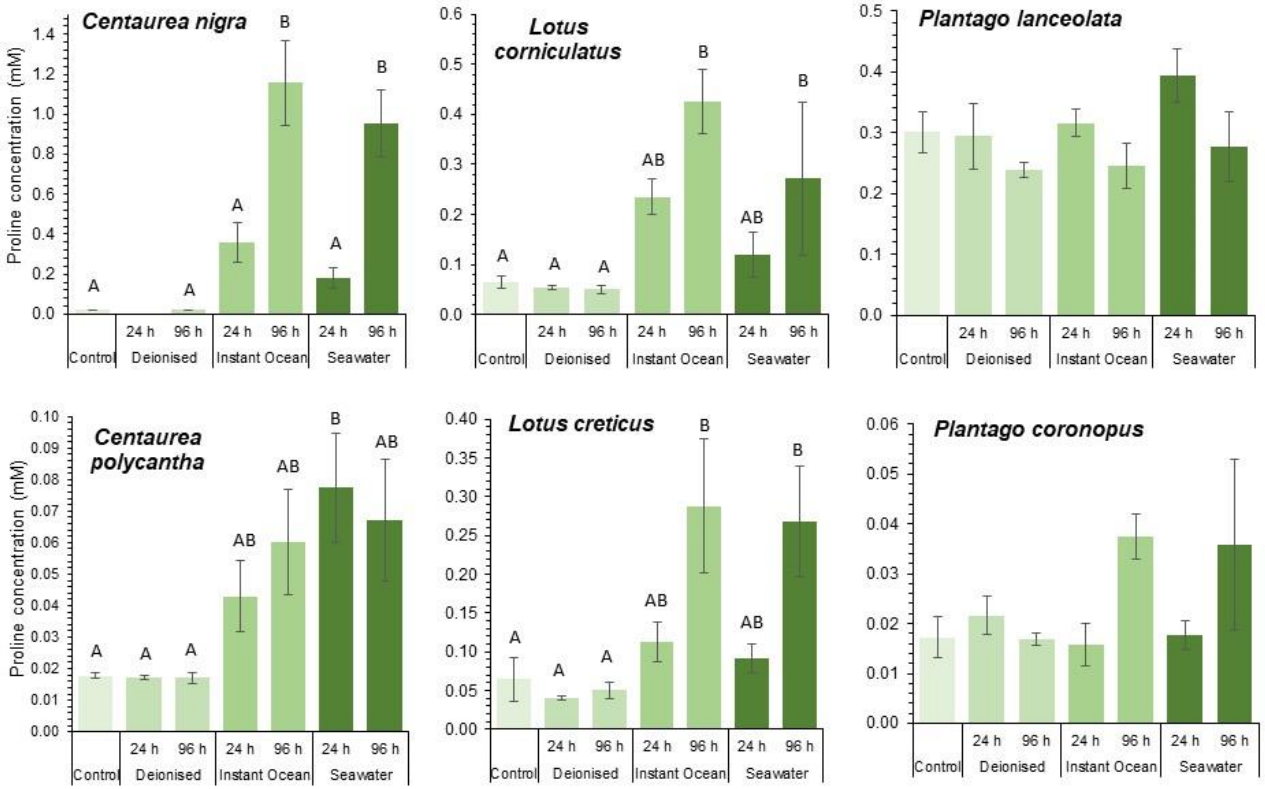

Figure 2. The effect of simulated seawater (marine aquarium salt solution 'Instant Ocean®' and natural 'Seawater') and freshwater ('Deionised') flooding on mean (±SE) leaf proline concentrations for six European coastal grassland species 3-d after root-zone immersion.

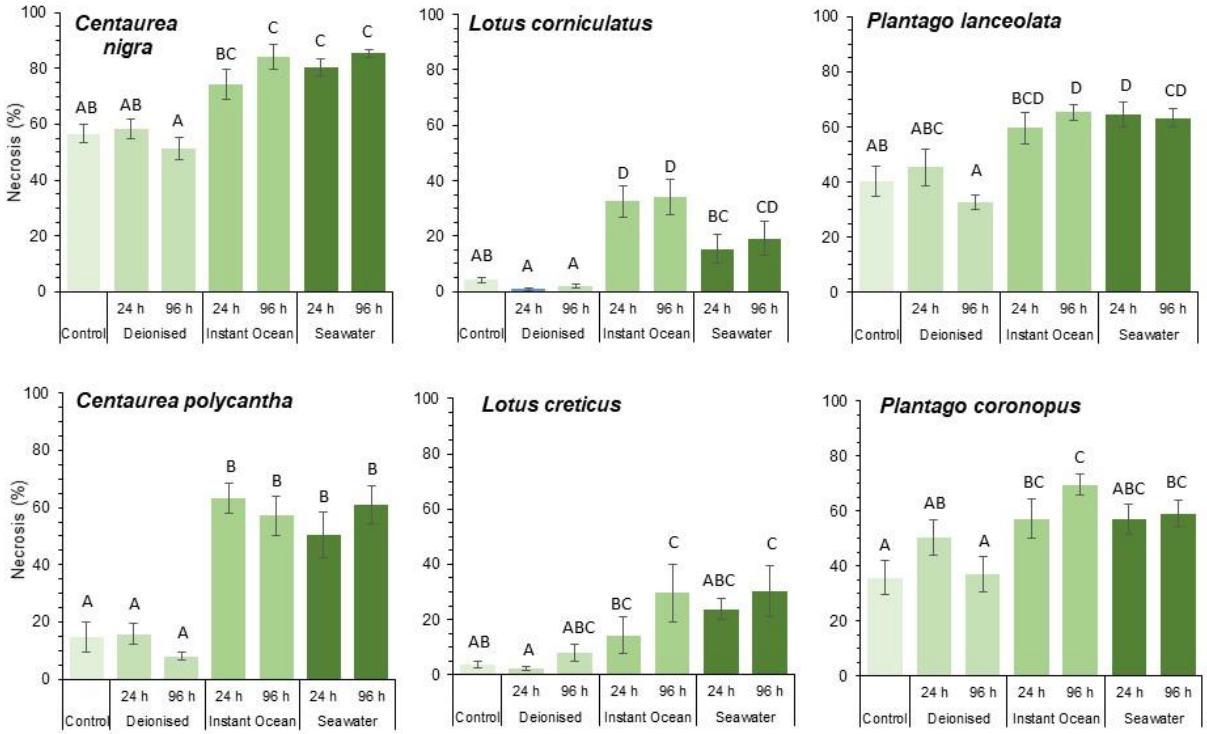

Figure 3. The effect of simulated seawater (marine aquarium salt solution 'Instant Ocean®' and natural 'Seawater') and freshwater ('Deionised') flooding on mean (±SE) above-ground tissue necrosis for six European coastal grassland species 35-d after root-zone immersion.

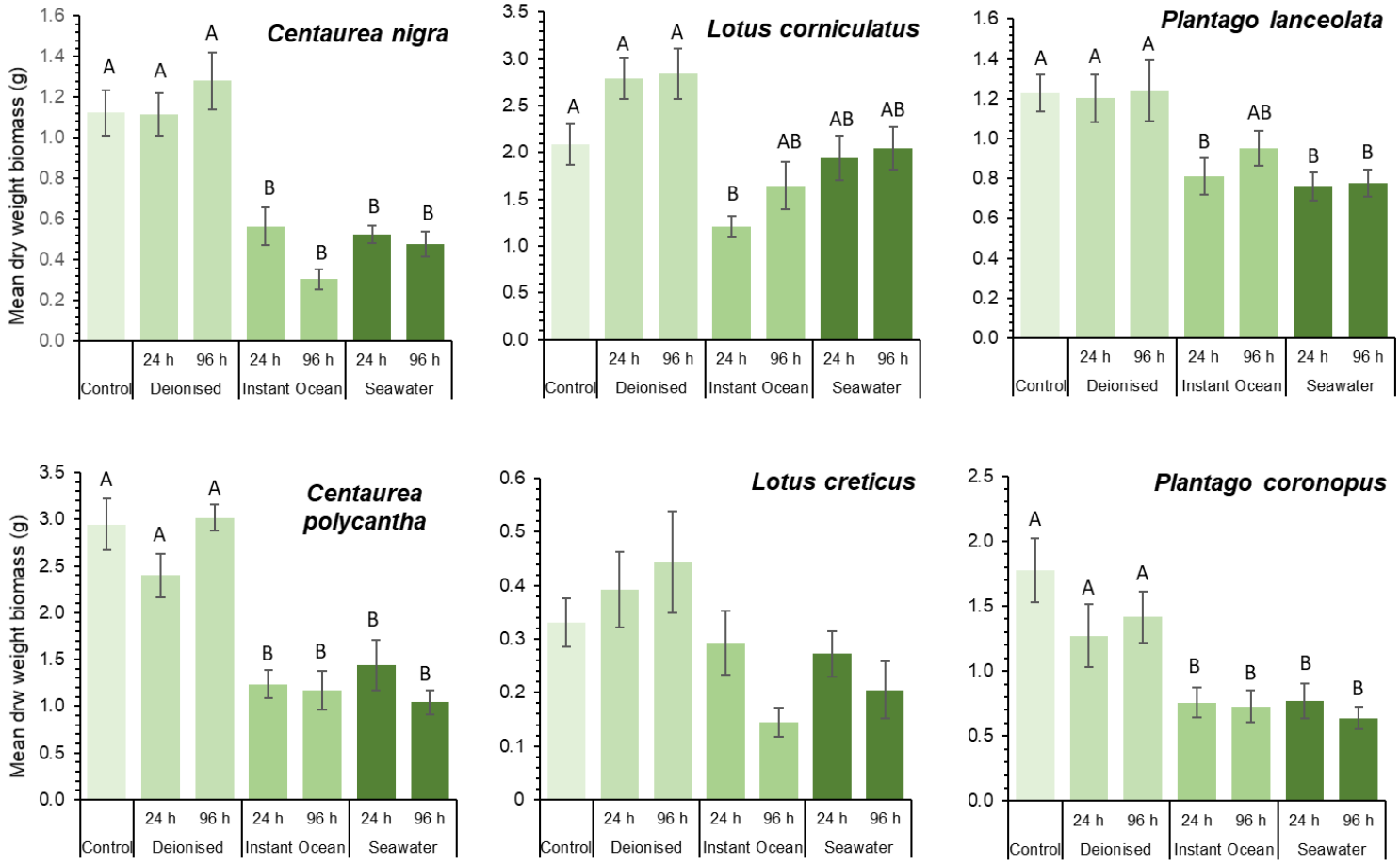

Figure 4. The effect of simulated seawater (marine aquarium salt solution 'Instant Ocean®' and natural 'Seawater') and freshwater ('Deionised') flooding on mean (±SE) total plant dry weight biomass for six European coastal grassland species 100-d after root-zone immersion.